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Early Innate Immunity to Bacterial Infection in the Lung Is Regulated Systemically by the Commensal Microbiota via Nod-Like Receptor Ligands

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The commensal microbiota is a major regulator of the immune system. The majority of commensal bacteria inhabit the gastrointestinal tract and are known to regulate local mucosal defenses against intestinal pathogens. There is growing appreciation that the commensal microbiota also regulates immune responses at extraintestinal sites. Currently, however, it is unclear how this influences host defenses against bacterial infection outside the intestine. Microbiota depletion caused significant defects in the early innate response to lung infection by the major human pathogen *Klebsiella pneumoniae*. After microbiota depletion, early clearance of *K. pneumoniae* was impaired, and this could be rescued by administration of bacterial Nod-like receptor (NLR) ligands (the NOD1 ligand MurNAcTri$_{DAP}$ and NOD2 ligand muramyl dipeptide [MDP]) but not bacterial Toll-like receptor (TLR) ligands. Importantly, NLR ligands from the gastrointestinal, but not upper respiratory, tract rescued host defenses in the lung. Defects in early innate immunity were found to be due to reduced reactive oxygen species-mediated killing of bacteria by alveolar macrophages. These data show that bacterial signals from the intestine have a profound influence on establishing the levels of antibacterial defenses in distal tissues.

Environmentally exposed surfaces in humans and other multicellular organisms are colonized by a vast number of microbes, collectively referred to as the commensal microbiota (1, 2). Humans are home to approximately 10^{13} to 10^{14} commensal bacteria, with the preponderance of these located in the gastrointestinal tract (3). The long evolutionary relationship between host and commensal microbiota means that these indigenous organisms influence many aspects of host physiology. Their importance has been demonstrated in numerous clinical studies and by using animal models, which show that disruption of host-commensal interactions is associated with a variety of diseases and conditions (1, 2, 4–14). These include cancer (8), chronic intestinal inflammation (12, 15), autoimmunity (14), and increased susceptibility to infection by bacteria, viruses, and parasites, both in the intestine and at extraintestinal sites (1, 4, 16–24). An underlying principal emerging from these studies is that the commensal microbiota is a major regulator of host immune function, and it is the disruption of this interaction that underlies many of these conditions. Therefore, understanding the interaction of the commensal microbiota and immune system is of major importance.

Given that the preponderance of commensal bacteria reside on the intestinal mucosa, most studies have focused on understanding how the microbiota regulates immunity at this site. This work has revealed that at the intestinal mucosa, pattern recognition receptors (PRRs) of the innate immune system are constantly engaged by the microbiota, and that this promotes maturation of the intestinal immune system and maintains intestinal homeostasis (12, 25). The adaptive immune system in the intestine is also regulated by the microbiota, with specific groups of commensal bacteria promoting the development of effector and regulatory T-cell populations (2). This includes induction of T_{H17} cells that fortify the mucosal barrier (26) and T_{REG} cells that dampen immune responses to prevent chronic inflammation (27, 28). Colonization by the microbiota also helps protect against intestinal infection. This occurs via numerous mechanisms, including the direct production of inhibitory molecules and depletion of nutrients by the microbiota to prevent the establishment of colonization and growth of potential pathogens (29–31). Additionally, the intestinal microbiota stimulates local innate production of antimicrobial peptides via PRRs to promote the killing of intestinal pathogens (17). Therefore, the commensal microbiota is crucial for optimal immune responses to intestinal pathogens.

In contrast, our understanding of how the commensal microbiota regulates immunity to infection at sites outside the intestine remains limited. The regulation of antiviral immunity at extraintestinal sites is perhaps the best characterized (32). Numerous studies have shown that in the absence of signals from commensal bacteria, the host is more susceptible to systemic and pulmonary viral infection (16, 22, 33). This has been ascribed to defects in the production of interferon by the innate immune system (16, 22) and reduced $CD4^+$ and $CD8^+$ T-cell generation during the adaptive antiviral response (33). Furthermore, the skin microbiota helps generate adaptive immune responses to protect against cutaneous infection by the parasite *Leishmania major* (11). Currently, and in contrast to other classes of pathogens, the understanding of how the microbiota regulates antibacterial immunity at extraintestinal sites is poor. It is known that in the absence of signals from commensal bacteria, mice more easily succumb to infection by a variety of bacterial pathogens, including *Listeria*

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monocytogenes and *Klebsiella pneumoniae* (9, 21, 23). Furthermore, it is known that killing of *Streptococcus pneumoniae* and *Staphylococcus aureus* by neutrophils from microbiota-depleted mice *ex vivo* is reduced (34). Therefore, currently it is broadly understood that the commensal microbiota helps protect against bacterial infection outside the intestine (9). What remain to be determined are the precise components of antibacterial immunity enhanced by the commensal microbiota and the demonstration that these components mediate protection against bacterial infection *in vivo*. Also, the nature of the signals that enhance extraintestinal antibacterial immunity and the origin of these signals need to be established. In this study, using a variety of *in vivo* and *ex vivo* models, I show that early defenses against respiratory infection by *K. pneumoniae*, a major lung pathogen, especially in patients receiving long-term antibiotic therapy, are enhanced by bacterial peptidoglycan. These cell wall components, recognized by the Nod-like receptors (NLRs) NOD1 and NOD2, originated from the intestine and enhanced the production of reactive oxygen species (ROS) in alveolar macrophages. Consequently, there was increased bacterial killing by these cells, and this was required to facilitate early bacterial clearance from the lung.

MATERIALS AND METHODS

Bacterial strains. *K. pneumoniae* (ATCC 43816) was cultured in LB broth with agitation at 37°C overnight.

Microbiota depletion. Mice were given broad-spectrum antibiotics (ampicillin, 1 g · liter^{-1}; neomycin sulfate, 1 g · liter^{-1}; metronidazole, 1 g · liter^{-1}; and vancomycin, 0.5 g · liter^{-1}) in drinking water for 10 to 14 days (25, 34, 35). Antibiotic therapy was stopped 3 days prior to infection.

Mouse models of infection. Six- to 8-week-old C57BL/6 mice (Charles River, United Kingdom) were anesthetized with isoflurane and inoculated intranasally with 1×10^5 CFU of *K. pneumoniae* in 50 μl of phosphate-buffered saline (PBS). To determine bacterial CFU, mice were sacrificed and lungs removed, homogenized in PBS, and plated on LB agar. To inhibit ROS production in the lung, mice were intranasally administered with 50 ml 0.5 mM *N*-acetyl-L-cysteine (NAC) (Sigma) 2 h prior to bacterial inoculation (36). Animal work was conducted in accordance with the Animal Scientific Procedures outlined by the UK Home Office regulations.

Isolation of alveolar macrophages. Alveolar macrophages were isolated as described in reference 37. Briefly, mice were sacrificed and immediately exsanguinated. Lungs were lavaged with 4 ml of 37°C Dulbecco's phosphate-buffered saline with 0.5 mM EDTA and without Ca^{2+} or Mg^{2+}. Cells then were pelleted and resuspended in RPMI supplemented with 2.5% (vol/vol) fetal bovine serum, 2 mM L-glutamine, 100 U · ml^{-1} penicillin, and 100 U · ml^{-1} streptomycin, and alveolar macrophages were allowed to adhere to a tissue culture flask for 1 h (37°C, 5% CO_2, vol/vol). Alveolar macrophage purity was approximately 90%.

Bacterial phagocytosis and killing assays. Bacterial phagocytosis and killing assays were performed essentially as described in references 38 to 40. Briefly, alveolar macrophages (1×10^5) were transferred to antibiotic-free Hanks balanced salt solution (HBSS) (plus Ca^{2+} and Mg^{2+}) and bovine serum albumin (BSA) and incubated with *K. pneumoniae* (1×10^6) opsonized in normal mouse serum at 37°C for 1 h. Gentamicin (10 mg · ml^{-1}) was added to kill extracellular bacteria, and the cells were washed in antibiotic-free HBSS. Macrophages then were either lysed using distilled water on ice or incubated for a further 2 h and then lysed. Bacterial viability is shown relative to the initial number of bacteria in the assay. To test the role of ROS in bacterial killing, alveolar macrophages were pretreated with 50 μM diphenyleneiodonium (DPI) for 30 min prior to incubation with *K. pneumoniae*. H_2O_2 production by alveolar macrophages was measured using the Amplex Red hydrogen peroxide assay kit (Molecular Probes) by following the manufacturer's instructions, as described previously (40). Briefly, bacterial killing assays were set up as described above, and after 3 h macrophages and bacteria were pelleted and the H_2O_2 concentration in the media determined.

Adoptive transfer of macrophages. Alveolar macrophages were isolated as described above from donor antibiotic-treated and non-antibiotic-treated mice. One day prior to bacterial inoculation, alveolar macrophages (1×10^5) were transferred from non-antibiotic-treated mice intranasally into recipient antibiotic-treated and non-antibiotic-treated mice. One day prior to bacterial inoculation, 1×10^5 alveolar macrophages also were transferred from antibiotic-treated mice intranasally into recipient antibiotic-treated and non-antibiotic-treated mice (41, 42).

qRT-PCR. cDNA was synthesized using a high-capacity cDNA reverse transcription kit according to the manufacturer's instructions (Applied Biosystems). Quantitative reverse transcription-PCRs (qRT-PCRs) were carried out as described before using SYBR green PCR master mix (Applied Biosystems) according to the manufacturer's instructions (43). Primers used in this study were the following: *Gapdh*, 5′-TGTGTCCGTC GTGGATCTGA-3′ and 5′-CCTGCTTCACCACCTTCTTGAT-3′; *Elastase*, CTGCTCCCATGAATGACAGTG and AGTTGCTTCTAGCCCAA AGAAC; *CathepsinD*, GCTTCCGGTCTTTGACAACCT and CACCAAG CATTAGTTCTCCTCC; *CathepsinG*, AGGGTTTCTGGTGCGAGAAG and GTTCTGCGGATTGTAATCAGGAT; *Cd45*, CAGAGCATTCCACG GGTATT and GGACCCTGCATCTCCATTTA; *il6*, GCCTCCTTGGGA CTGATGCT and AGTCTCCTCTCCGGACTTGTG; *tnfa*, CCCAGGCA GTCAGATCATCTTC and AGCTGCCCCTCAGCTTGA. Differential expression was calculated using the $\Delta\Delta C_T$ method (C_T stands for threshold cycle) and is shown relative to the level for *Gapdh* ± standard errors of the means (SEM).

Preparation of PRR ligands. Lipopolysaccharide (LPS) was purified from *H. influenzae* (H636) by hot phenol-water extraction as described previously (43). Lipoteichoic acid (LTA) was purified from *S. aureus* (Newman) by *n*-butanol extraction as described previously (44, 45). Briefly, cells were resuspended in butanol-water (1:1, vol/vol) and stirred at room temperature for 30 min. The aqueous phase was isolated after centrifugation, concentrated, dialyzed overnight, and lyophilized. Bacterial DNA, as a source of unmethylated CpG DNA, was isolated as previously described (46). *Escherichia coli* (DH5α) was grown overnight in LB broth. Bacteria were pelleted and resuspended in 10 mM Tris, 50 mM EDTA, pH 8.0, supplemented with lysozyme (0.5 mg · ml^{-1}) and proteinase K (2 mg · ml^{-1}), and incubated for 2 h. SDS then was added to a final concentration of 1% (vol/vol), and incubation continued for a further 3 h at 50°C. DNA was isolated from this lysate using phenol-chloroform-isoamyl alcohol extraction. Muramyl dipeptide (MDP) and $MurNAcTri_{DAP}$ were synthesized by *in vitro* reconstruction of the peptidoglycan biosynthetic pathway as described previously (47).

Measurement of reactive oxygen species. The production of reactive oxygen species was assayed using the Amplex Red hydrogen peroxide assay kit (Molecular Probes) to monitor the production of H_2O_2. For *in vivo* samples, H_2O_2 levels in undiluted bronchoalveolar lavage fluid were measured.

Statistical analysis. Analysis of variance (ANOVA) was used to compare multiple groups, with *post hoc* Turkey's test or Dunnett's test used as appropriate. The unpaired Student's *t* test was used to compare two groups. *P* values of ≤0.05 were considered significant (GraphPad Prism 4).

RESULTS

The commensal microbiota enhances early innate defenses to bacterial infection in the lung. To determine the role of the commensal microbiota in regulating antibacterial immunity outside the intestine, a model of bacterial infection of the lung was used. Mice were treated with broad-spectrum antibiotics (ampicillin, neomycin, metronidazole, and vancomycin) in their drinking water for >10 days, an established protocol that depletes the commensal bacteria in the intestine and upper airway (25, 33–35), and

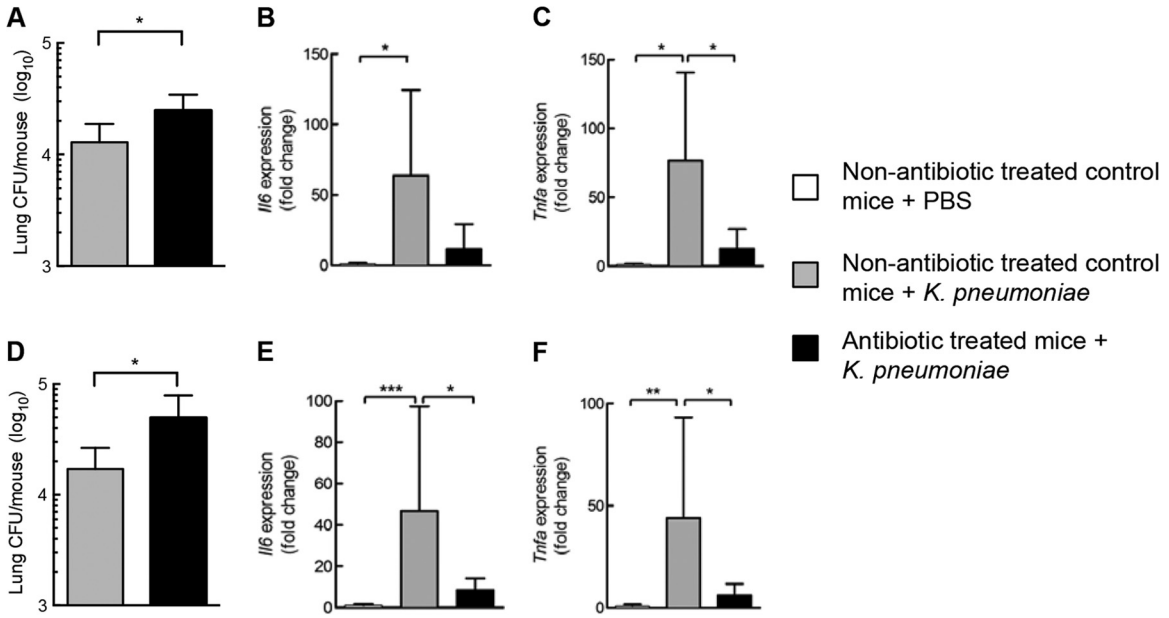

FIG 1 Commensal microbiota promotes early innate clearance of bacterial infection in the lung. Wild-type mice treated with antibiotics and non-antibiotic-treated controls were intranasally inoculated with *K. pneumoniae* 3 days postantibiotic cessation. Mice were sacrificed 3 h (A to C) and 6 h (D to F) postinoculation, bacterial burden in the lungs was quantified, and RNA was isolated from lung tissue to analyze relative mRNA levels by qRT-PCR. Tnfa, tumor necrosis factor alpha. Values represent five independent determinations ± SEM. Statistical significance was determined by *t* test. *, $P < 0.05$.

then intranasally inoculated with *K. pneumoniae* 3 days postantibiotic cessation. Compared to the level in non-antibiotic-treated control mice, there was a significant increase in the bacterial burden in the lungs of mice treated with antibiotics 3 h postinoculation (Fig. 1A). At this time point there also was decreased expression of interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) in the lung tissue of antibiotic-treated mice compared to that of non-antibiotic-treated mice, cytokines that are known to be part of the early inflammatory response to bacterial infection (48) (Fig. 1B and C). There was also a significant increase in bacterial burden in the lung at 6 h postinoculation in antibiotic-treated mice compared to that in non-antibiotic-treated control mice (Fig. 1D), and this again correlated with a significant reduction in the expression of IL-6 and TNF-α in the lung (Fig. 1E and F). Collectively, these data demonstrate that antibiotic treatment results in a dampened inflammatory response to infection in the lung and impaired bacterial clearance.

NOD1 and NOD2 ligands derived from the intestine restore early innate defenses to bacterial infection of the lung. The stimulation of PRRs by the microbiota in the intestine promote local innate defenses to bacterial infection at this site (17), and PRR ligands restore defective adaptive immunity to viral infection in the lung after microbiota depletion (33). Previous work also has shown that bacterial PRR ligands from the commensal microbiota are present in the circulation of normal healthy mice and humans (34, 49, 50). These PRR ligands bathe nonmucosal tissues and can enhance the antibacterial activity of bone marrow-derived neutrophils in *ex vivo* killing assays (34). This led to the hypothesis that the microbiota is a source of PRR ligands that promote innate immunity to bacterial infection in the lung. To test this, mice were treated with antibiotics, which reduces both the burden of commensal bacteria and also the concentration of PRR ligands in the circulation (25, 34), and then were orally gavaged with PRR ligands 48 and 24 h prior to intranasal inoculation with *K. pneumoniae* (33). Six hours postinoculation, the lung burdens of *K. pneumoniae* in antibiotic-treated mice gavaged with either the bacterial Toll-like receptor (TLR) ligand LPS (TLR4 ligand), Pam_3CSK_4 (P3C) (TLR2/1), or CpG (TLR9) were significantly higher than those of non-antibiotic-treated mice, showing that TLR ligands could not rescue any defects in early bacterial clearance caused by antibiotic treatment (Fig. 2A). In contrast, antibiotic-treated mice gavaged with bacterial peptidoglycan recognized by NLRs (either MDP recognized by NOD2 or $MurNAcTri_{DAP}$ recognized by NOD1) had *K. pneumoniae* burdens that were not significantly different from those of non-antibiotic-treated mice with a microbiota (Fig. 2A). Furthermore, increasing the degree of TLR stimulation by increasing the amount of LPS mice were treated with still was unable to rescue defects in early bacterial clearance, whereas reduced NLR stimulation still was sufficient to restore early innate defenses (Fig. 2A). These data demonstrate that NLR, but not TLR, stimulation via the intestine is sufficient to restore early innate immune responses to bacterial infection in the lung after antibiotic treatment. In addition to restoring early clearance, NLR, but not TLR, stimulation also was able to rescue defects in IL-6 production after antibiotic treatment (Fig. 2B). This supports the hypothesis that microbiota-derived PRR ligands exert a systemic tonic priming effect on the innate immune system.

The intestine is not the only mucosal barrier site colonized by commensal bacteria, and other studies have shown that the upper airway microbiota influences lung defenses against viral infection (42). Given this role in regulating lung immunity and that antibiotic administration in drinking water depletes both the intestinal and upper airway microbiota (33), I investigated the hypothesis that the upper airway is also a source of PRR ligands that promote the early clearance of bacteria in the lung. Mice treated with antibiotics and nontreated control mice were intranasally inoculated

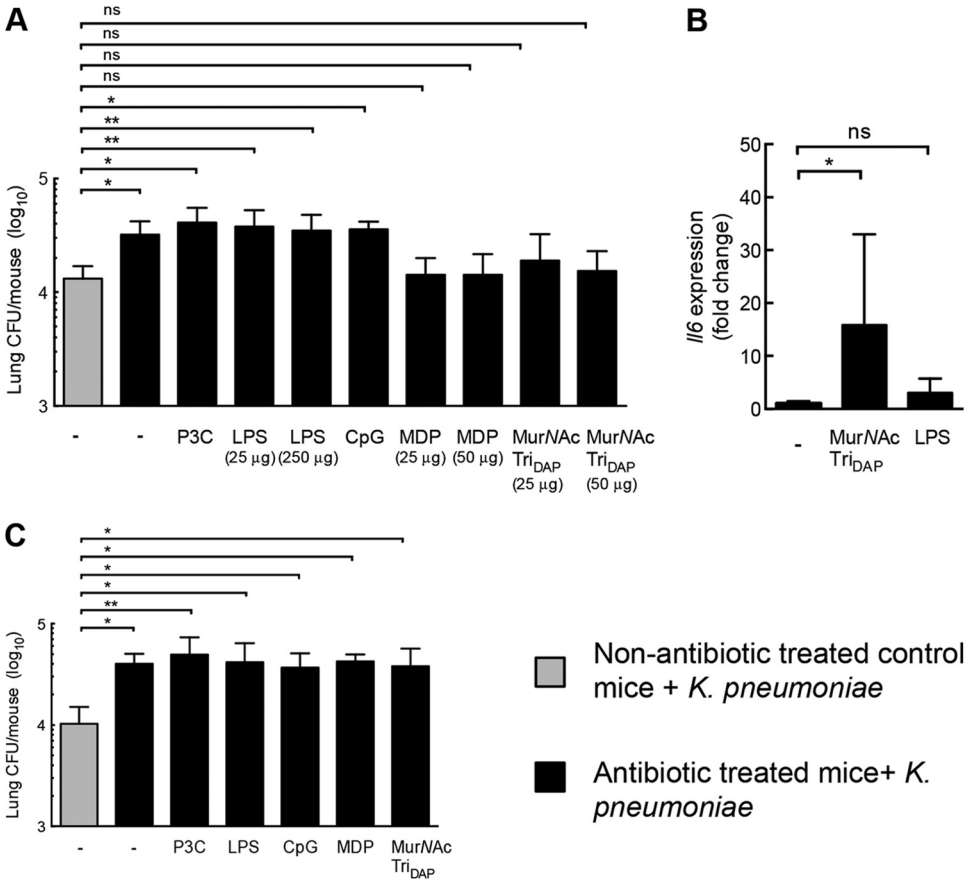

FIG 2 Oral administration of NLR ligands restores early antibacterial defenses in the lung after commensal microbiota depletion. (A) Wild-type mice treated with antibiotics and non-antibiotic-treated controls were gavaged with either P3C (50 μg), LPS (25 μg or 250 μg), CpG (25 μg), MDP (50 μg or 25 μg), or MurNAcTri$_{DAP}$ (50 μg or 25 μg) 48 h and 24 h prior to infection with *K. pneumoniae*. Six hours postinoculation, mice were sacrificed and bacterial burden in the lungs was quantified. (B) Wild-type mice were treated with antibiotics and gavaged with either LPS (25 μg), MurNAcTri$_{DAP}$ (50 μg), or PBS (as a control) 48 h and 24 h prior to infection with *K. pneumoniae*. Six hours postinoculation, mice were sacrificed and RNA isolated from lung tissue to analyze relative mRNA levels by qRT-PCR. (C) Wild-type mice treated with antibiotics and non-antibiotic-treated controls were intranasally administered either P3C (50 μg), LPS (25 μg), CpG (25 μg), MDP (50 μg), or MurNAcTri$_{DAP}$ (50 μg) 48 h and 24 h prior to infection with *K. pneumoniae*. Six hours postinoculation, mice were sacrificed and bacterial burden in the lungs was quantified. Values represent five independent determinations ± SEM. Statistical significance was determined using one-way ANOVA with *post hoc* Dunnett's test or by *t* test. *, $P < 0.05$; **, $P < 0.01$; ns, not significant.

with either LPS, P3C, CpG, MDP, or MurNAcTri$_{DAP}$ 48 and 24 h prior to intranasal inoculation with *K. pneumoniae*. Bacterial burdens in the lung 6 h postinoculation all were significantly higher than those of non-antibiotic-treated mice (Fig. 2C), demonstrating that intranasal PRR ligands cannot restore early bacterial clearance in the lung. Taken together, these data show that NLR ligands originating from the intestine, but not the upper airway, enhance early innate mechanisms of bacterial clearance in lung tissue.

Alveolar macrophages from non-antibiotic-treated animals restore innate defenses to bacterial infection in the lung after microbiota depletion. While numerous studies have shown that the commensal microbiota helps protect the host against infection, the antibacterial effector mechanisms and cell types programmed by the microbiota that mediate this protection are poorly defined. Early bacterial clearance from the lung requires alveolar macrophages and, as infection progresses, recruited neutrophils (51, 52). In previous work, the microbiota was shown to have a systemic effect on neutrophils in the bone marrow and enhance their bacterial killing capacity (34). However, I found that bacterial burdens at early time points (3 and 6 h postinoculation with *K. pneumoniae*) in the lungs of antibiotic-treated and non-antibiotic-treated mice depleted of neutrophils via antibody treatment were equivalent (data not shown). This indicated that any enhancements in early bacterial clearance in the lung due to the microbiota were independent of neutrophils. qRT-PCR analysis of lung tissue also showed that the numbers of alveolar macrophages in the lung were equivalent in antibiotic- and non-antibiotic-treated mice (data not shown). Therefore, I investigated whether reduced bacterial clearance after microbiota depletion was due to functional defects in alveolar macrophages. To do this, I took an adoptive transfer approach, and alveolar macrophages from both antibiotic-treated and non-antibiotic-treated mice were isolated. Alveolar macrophages from antibiotic-treated mice then were transferred into both antibiotic-treated and non-antibiotic-treated recipient mice. Similarly, alveolar macrophages from non-antibiotic-treated mice were transferred into antibiotic-treated and non-antibiotic-treated recipients. All four groups of mice then were infected with *K. pneumoniae*, and lung bacterial burden at 6 h postinoculation was determined. In control, non-

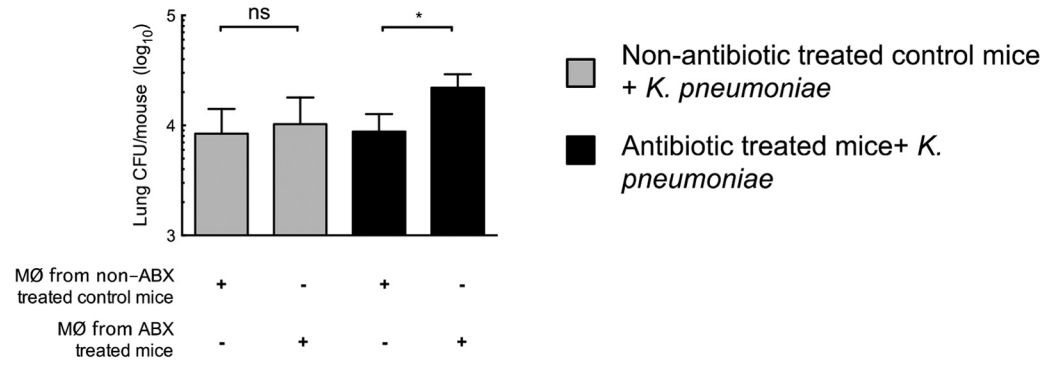

FIG 3 Alveolar macrophages (MØ) from non-antibiotic-treated mice rescue antibacterial defenses in the lung in the absence of the commensal microbiota. Wild-type mice treated with antibiotics (ABX) and non-antibiotic-treated controls were adoptively transferred intranasally with alveolar macrophages from mice of the indicated origins 24 h prior to infection with *K. pneumoniae*. Six hours postinoculation, mice were sacrificed and bacterial burden in the lungs was quantified. Values represent five independent determinations ± SEM, and statistical significance was determined by *t* test. ns, not significant; *, $P < 0.05$.

antibiotic-treated mice that are able to clear infection normally, bacterial burden was equivalent after they had received alveolar macrophages from either non-antibiotic-treated or antibiotic-treated mice (Fig. 3A). In contrast, the bacterial burden in the lungs of mice treated with antibiotics, which are defective in clearance of bacteria, was significantly reduced upon transfer of macrophages from non-antibiotic-treated mice compared to the level for transfer of macrophages from mice treated with antibiotics (Fig. 3A). These data show that restoration of a population of alveolar macrophages from mice with a microbiota into the lungs of mice treated with antibiotics is sufficient to restore defects in early bacterial clearance caused by antibiotic treatment.

ROS-mediated bacterial killing by alveolar macrophages is impaired in the absence of signals from the commensal microbiota. Alveolar macrophages have a variety of mechanisms to kill bacteria, including lysosomal proteases, antimicrobial peptides, and reactive oxygen species (53). To determine how the microbiota increases the ability of alveolar macrophages to clear bacteria from the lung, alveolar macrophages from antibiotic-treated and non-antibiotic-treated mice were isolated and their ability to kill *K. pneumoniae ex vivo* was assessed. After incubation of *K. pneumoniae* with alveolar macrophages for 1 h, the levels of viable intracellular bacteria were similar between macrophages from antibiotic-treated mice and nontreated controls (Fig. 4A). Furthermore, pretreatment of mice with bacterial NLR or TLR ligands by oral gavage prior to macrophage isolation had no effect on the levels of intracellular bacteria in alveolar macrophages after 1 h of incubation with *K. pneumoniae* (Fig. 4A). Data at this early time point suggest that the uptake of bacteria by alveolar macrophages is unaffected by antibiotic treatment. In contrast, after 3 h of incubation of *K. pneumoniae* with alveolar macrophages, the number of viable bacteria recovered from macrophages isolated from antibiotic-treated mice was significantly higher than that from macrophages isolated from non-antibiotic-treated controls (Fig. 4B). These data demonstrate that antibiotic treatment does not affect bacterial uptake but does reduce the bacterial killing capacity of alveolar macrophages. This reduction in bacterial killing by alveolar macrophages could be rescued by pretreatment of mice with MDP or $MurNAcTri_{DAP}$ but not LPS, and this also was dependent on ROS (Fig. 4B), which is in agreement with *in vivo* data showing that bacterial NLR ligands, but not TLR ligands, restore early innate clearance of bacteria in the lung after antibiotic treatment (Fig. 2A).

Reactive oxygen species are important for killing bacterial pathogens in the lung (54), and pretreatment of alveolar macrophages with DPI, an inhibitor of reactive oxygen species generation, resulted in decreased bacterial killing and also abrogated differences in bacterial killing between macrophages isolated from antibiotic-treated and nontreated mice (Fig. 4B). This showed that ROS are required for killing of *K. pneumoniae* and that the microbiota enhances reactive oxygen species-mediated bacterial killing by alveolar macrophages. To ascertain if the microbiota promotes reactive oxygen species production, alveolar macrophages were isolated from antibiotic-treated and non-antibiotic-treated mice and incubated with *K. pneumoniae*, and the levels of H_2O_2 produced, as a marker of reactive oxygen species, were measured. Alveolar macrophages from antibiotic-treated mice incubated with *K. pneumoniae* produced significantly less H_2O_2 (by 57.8% ± 2.9%) relative to macrophages from non-antibiotic-treated mice incubated with *K. pneumoniae* (Fig. 4C). In accordance with *in vivo* data showing that only NLR ligands can restore early innate defenses to bacterial infection in the lung after microbiota depletion (Fig. 2A), oral administration of MDP or $MurNAcTri_{DAP}$, but not LPS, to antibiotic-treated mice 48 and 24 h prior to macrophage isolation restored H_2O_2 levels to those of non-antibiotic-treated animals (Fig. 4C).

Taken together, these data suggest that the microbiota does not influence bacterial uptake by alveolar macrophages but promotes intracellular bacterial killing by increasing reactive oxygen species generation. The expression of the antimicrobial proteases elastase, cathepsin G, and cathepsin D was not significantly different between macrophages isolated from antibiotic-treated and non-treated mice (Fig. 4E and F), supporting the hypothesis that the major antibacterial effector mechanism enhanced by the microbiota is ROS production.

The commensal microbiota and NLR ligands regulate ROS production *in vivo*, and this is required for early clearance of bacteria from the lung. Given the reduction in reactive oxygen species production in alveolar macrophages and concomitant reduction in bacterial killing by these cells after antibiotic treatment of mice, as well as the ability of NLR ligands from the intestine to restore reactive oxygen production and bacterial killing by alveo-

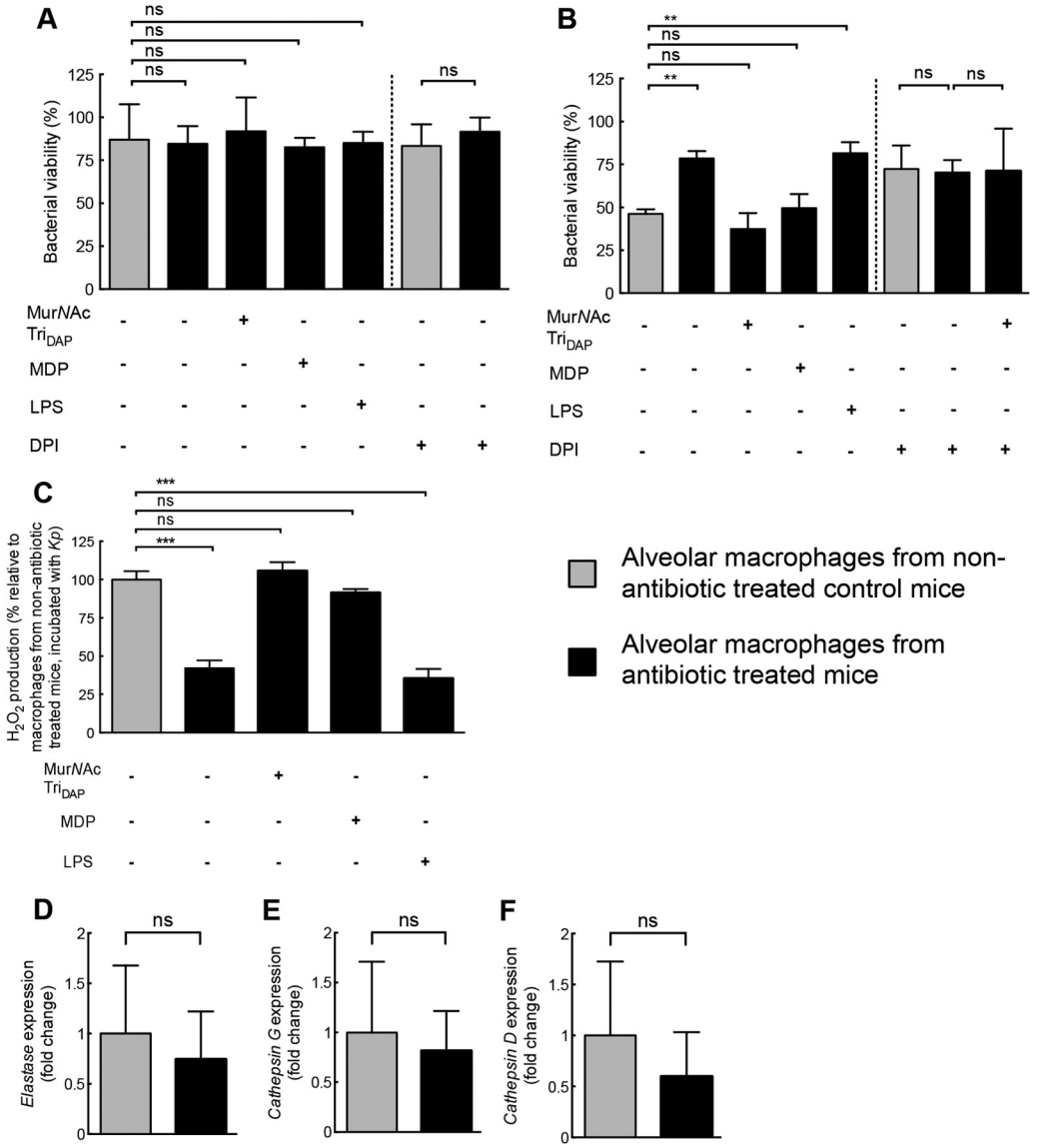

FIG 4 ROS-mediated bacterial killing by alveolar macrophages is enhanced by the commensal microbiota and NLR ligands. (A and B) Alveolar macrophages isolated from wild-type mice treated with antibiotics and non-antibiotic-treated controls were incubated with *K. pneumoniae* for 1 h (A) and 3 h (B), and bacterial viability then was determined and expressed relative to the initial number of bacteria in the assay (approximately 1×10^6). The indicated groups of mice were gavaged with LPS (25 μg), MDP (50 μg), or MurNAcTri$_{DAP}$ (50 μg) 48 h and 24 h prior to macrophage harvest. For the indicated experiments, macrophages were pretreated with DPI (50 μM) for 30 min prior to incubation with *K. pneumoniae*. Values represent five independent determinations ± SEM. Statistical significance was determined using one-way ANOVA with *post hoc* Turkey's test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (C) Alveolar macrophages from wild-type mice treated with antibiotics and non-antibiotic-treated controls were harvested and incubated with *K. pneumoniae* for 3 h, and H_2O_2 produced by alveolar macrophages was measured. The indicated groups of mice were gavaged with LPS (25 μg), MDP (50 μg), or MurNAcTri$_{DAP}$ (50 μg) 48 h and 24 h prior to macrophage harvest. Values represent five independent determinations ± SEM. Statistical significance was determined using one-way ANOVA with *post hoc* Dunnett's test. ns, not significant; ***, $P < 0.001$. (D to F) Alveolar macrophages from wild-type mice treated with antibiotics and non-antibiotic-treated controls were harvested and RNA isolated to analyze relative mRNA levels by qRT-PCR. Values represent five independent determinations ± SEM. Statistical significance was determined by *t* test. ns, not significant.

lar macrophages, I investigated the role played by reactive oxygen species in early bacterial clearance from the lung *in vivo* and how this is regulated by the microbiota and NLR ligands. To do this, the indicated groups of mice were intranasally administered *N*-acetyl-L-cysteine (NAC) 2 h prior to intranasal inoculation with *K. pneumoniae*. NAC is an oxidant scavenger, and its intranasal administration is an established method to reduce reactive oxygen species levels in the lung (36). Importantly, at the concentrations used in this study, NAC has no direct effect on *K. pneumoniae* growth (55). In control mice not treated with NAC, antibiotic treatment resulted in significantly higher *K. pneumoniae* burdens in the lung compared to that of non-antibiotic-treated mice 6 h postinoculation (Fig. 5A). After intranasal NAC administration, the burden of *K. pneumoniae* in the lungs of both antibiotic-treated and nontreated groups was significantly higher than that of control non-antibiotic-treated mice not administered NAC

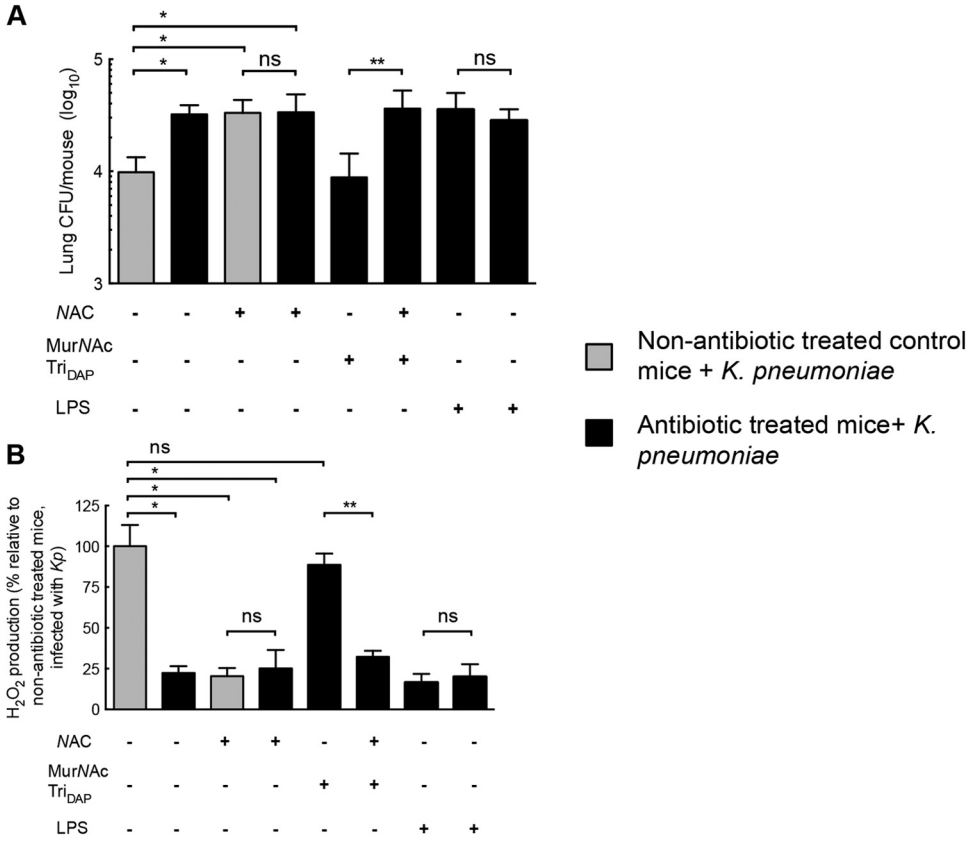

FIG 5 Early bacterial clearance from the lung requires ROS, and ROS production *in vivo* is enhanced by the commensal microbiota and NLR ligands. (A and B) Wild-type mice treated with antibiotics and non-antibiotic-treated controls were intranasally administered NAC or vehicle control 2 h prior to infection with *K. pneumoniae*. Six hours postinoculation, mice were sacrificed and bacterial burden (A) and H_2O_2 (B) in the lungs quantified. The indicated groups of mice were gavaged with MurNAcTri$_{DAP}$ (50 μg) 48 h and 24 h prior to infection with *K. pneumoniae*. Values represent five independent determinations ± SEM. Statistical significance was determined using one-way ANOVA with *post hoc* Turkey's test. ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

(Fig. 5A). Crucially, there was no significant difference in bacterial clearance between antibiotic-treated and nontreated mice after NAC treatment, suggesting that microbiota-mediated enhancement of early bacterial clearance requires reactive oxygen species production. This is in agreement with *ex vivo* data demonstrating the requirement for ROS for microbiota-mediated enhancement of *K. pneumoniae* killing by alveolar macrophages. Furthermore, the ability of NLR ligands to restore host defenses in the absence of the microbiota also was inhibited by NAC treatment (Fig. 5A), whereas TLR ligands played no apparent role. Because of this important role of reactive oxygen species in mediating clearance, the levels of H_2O_2 *in vivo* were measured. H_2O_2 production in the lungs of infected mice pretreated with antibiotics was significantly reduced (by 77.6% ± 2.5%) relative to that of non-antibiotic-treated mice infected with *K. pneumoniae*, showing that the microbiota enhances reactive oxygen species production *in vivo* (Fig. 5B). Prior intranasal administration of NAC to both antibiotic- and non-antibiotic-treated mice significantly reduced H_2O_2 production compared to that of non-antibiotic-treated mice that had not been treated with NAC (Fig. 5B). There was no difference in the levels of H_2O_2 production between antibiotic and non-antibiotic-treated mice administered NAC (Fig. 5B). This correlates with bacterial burdens in the lung of antibiotic-treated and nontreated mice where microbiota-mediated enhancement of early bacterial clearance was lost after inhibition of reactive oxygen species by NAC (Fig. 5A). Oral administration of a bacterial NLR ligand, but not TLR ligand, restored H_2O_2 production in antibiotic-treated mice to levels equivalent to those of non-antibiotic-treated mice, and this too was inhibited by pretreatment with NAC (Fig. 5B). These data demonstrate that reactive oxygen species play a significant role in the early clearance of bacterial pathogens from the lung and that reactive oxygen species-mediated clearance is promoted by the microbiota. Bacterial NLR ligands, but not TLR ligands, from the gastrointestinal tract are sufficient to restore reactive oxygen-mediated bacterial clearance in the lung, providing further strong evidence for the systemic role of intestinal microbiota-derived NLR ligands in promoting innate immune responses to bacterial infection at tissues distal to the intestine.

DISCUSSION

Macrophages and neutrophils provide an effective way for the host to rapidly deploy powerful antimicrobial effectors, such as proteases, antimicrobial peptides, and reactive oxygen species, in a targeted manner to control infection and maintain tissue homeostasis (56). These antimicrobial effectors, however, come at a cost, both in the energetic input required for their production and mobilization and also because they act indiscriminately and can be extremely damaging to host tissues (57, 58). Thus, an appropriate immune set-point must be established with adequate production,

deployment, and functioning of innate cells to facilitate control of a given pathogenic threat without damaging exuberance and profligate use of resources. An implicit assumption made when considering innate immunity and inflammation has been that the host is the major regulator that establishes this set-point (56, 59), and that microbial influences on this are restricted to fine-tuning innate cell function locally in the vicinity of the mucosa (60). It is now becoming apparent that this localized view is incorrect and that commensal microbes in the intestine exert a systemic influence on effector cells of the innate immune system at extraintestinal sites, and that this contributes to the establishment of the innate immune set-point (8, 16, 22, 33, 34). The mechanistic basis for these distal influences, the precise cellular functions in innate cells regulated systemically by commensal bacteria, and the impact this has on host defenses to bacterial infection outside the intestine have been incompletely characterized. Previous studies have shown increased mortality from bacterial infection in the lung in the absence of the microbiota (21, 61), but the specific immune defects that cause this are poorly understood. Data presented here show that the antibacterial activity of alveolar macrophages is compromised in the absence of the commensal microbiota, leading to defects in early bacterial clearance from the lung, which can be restored by administration of bacterial NLR ligands via the gastrointestinal tract. This builds on previous work showing that microbiota-derived NOD1 ligands enhance the antibacterial activity of neutrophils in bone marrow (34). Furthermore, in this study and in contrast to previous work (34), I was able to show that the antibacterial effector mechanism enhanced by the microbiota and required for efficient clearance of bacteria from the lung was the production of reactive oxygen species in alveolar macrophages.

Alveolar macrophages are key sentinels that constantly patrol and monitor lung tissue (51, 52). These cells are long-lived, with approximately 40% of alveolar macrophages replaced per year in a healthy murine lung, and are the first line of defense against respiratory pathogens (62). Tissue-specific cues ensure alveolar macrophages are ideally suited to their role in the lung; however, local reprogramming in response to chronic inflammation or infection allows adaptation to environmental changes (62). For example, after the resolution of lung infection by influenza, alveolar macrophages undergo enduring changes, producing reduced levels of inflammatory cytokines and increased levels of anti-inflammatory cytokines, such as IL-10, when restimulated by TLR ligands after infection (19). Data from the current study show that, in addition to local signals, the antibacterial activity of alveolar macrophages is programmed systemically by signals from the intestine. This systemic effect of intestine-derived signals on lung function fits with recent work showing that shifts in the composition of the intestinal microbiota cause changes in alveolar macrophages that increase allergic inflammation in the airway (41). The role of pattern recognition receptors was not investigated in that study, and changes in alveolar macrophage function were shown to be due to increased prostaglandin E_2 levels in the circulation (41). Additionally, another study has shown that defects in migration to draining lymph nodes and reduced production of IL-1β by lung dendritic cells lead to reduced adaptive immune responses to influenza virus infection after microbiota depletion, and that this could be corrected by intrarectal administration of TLR ligands (33).

The importance of lung integrity for gaseous exchange means that the production of inflammatory mediators and any molecule that could cause tissue damage by alveolar macrophages is severely restrained. Reactive oxygen species are a crucial antibacterial effector mechanism in the lung, but as they act nonspecifically, they have the potential to cause significant damage. Thus, a variety of detoxification mechanisms operate to mitigate their deleterious effects (63). Data presented here show that in addition to detoxification, the host meters the production of reactive oxygen species in response to microbiota-derived signals. This supports a model of host defense whereby the levels of ROS production are continually gauged to facilitate host control of bacteria, whether they are commensal bacteria at the mucosa or acquired pathogens that gain entry into normally sterile tissues or tissues that can tolerate only a very small number of bacteria, such as the lung, while minimizing ROS production and concomitant tissue damage. Taken together, previous studies (19, 62) and data presented here show that macrophages in the lung assimilate information from various local and systemic cues and modify their function accordingly in order to maintain tissue homeostasis while maximizing local host defenses. As lung infection remains a major cause of mortality worldwide, this underappreciated flexibility is of significant therapeutic potential, as it may be possible to reprogram lung defenses to improve immune responses to clear infection.

The localized influence of commensal bacteria on immunity to infection at the barrier site they colonize is increasingly well characterized, especially in the intestine. Outside the intestine, it has also been shown that skin commensals regulate local T-cell-mediated immunity to cutaneous *Leishmania major* infection via IL-1 signaling (11), and upper airway commensals regulate immunity to viral infection in the lower airway (42). Furthermore, studies have also shown that repeated intranasal administration of a combination of both bacterial and fungal ligands to mice colonized by commensal bacteria provides additional local stimulation that enhances survival during bacterial lung infection (64). In the current study, defects in bacterial clearance from the lung due to microbiota depletion could be rescued only by NLR ligands originating from the intestine and not the upper airway. This suggests that under basal conditions the intestinal microbiota, not the airway microbiota, play a dominant role in establishing the levels of early antibacterial immunity in the lung. Furthermore, it shows that commensal bacteria at one barrier site can regulate antibacterial immunity at another, distal barrier site. As the current study focused on the very immediate response to infection, this does not preclude the possibility that the upper airway microbiota, or TLR ligands, regulate other aspects of lung immunity important at later time points during infection. For example, other studies have shown that in the absence of signals from the microbiota, there is increased mortality during bacterial lung infection, and this could be rescued by LPS administration either in the drinking water or via intraperitoneal injection (21, 61). These studies analyzed later time points in infection than this study and did not address the role of NLRs, but they do raise the possibility that TLR ligands regulate other components of lung immunity important during the later stages in lung infection. Further work to understand how immunity at one barrier site is programmed by signals from both proximal and distal commensal populations is required to address this. The mechanistic basis as to why early bacterial clearance in the lung could be rescued only by NLR ligands from the intestine and not the upper airway currently is unclear but no doubt reflects the central role the intestinal microbiota has evolved to play in regulating the immune system. One possible explanation is the

requirement of an intermediate signal between commensal stimulation of NLRs in the intestine and enhanced macrophage function in the airway. In contrast to TLRs, whose activation is tightly restrained in the intestine (65), NLRs are expressed in the intestinal mucosa and are activated by resident commensals (66), and this could result in the production of a signal originating from the intestinal mucosa that has a systemic effect on host lung function. Alternatively, peptidoglycan from the intestinal microbiota is found systemically in nonmucosal tissues of healthy mice and humans (including blood, spleen, and bone marrow) (34, 49, 50), and this disseminated peptidoglycan may activate underlying lung tissue to regulate alveolar macrophage function.

The priming of alveolar macrophage function in the lung and neutrophil function in the bone marrow via recognition of microbiota-derived peptidoglycan by NLRs is part of a wider phenomenon of immune recognition of PRR ligands under homeostatic conditions. PRRs originally were thought to sense the presence of infectious microbes and promote pathogen clearance (67), but data from this study and numerous others (16, 22, 25, 33, 34, 68, 69) show basal activation of PRRs by the microbiota in both mucosal and nonmucosal tissues in the absence of infection. This is important for the development of the immune system (6, 13), facilitates colonization by the commensal microbiota (69), prevents chronic inflammation (25), and enhances killing of pathogens by innate cells (34). Recent studies also have shown that basal stimulation of the innate immune system by the microbiota via PRRs and their ligands promotes hematopoiesis (18, 24), increasing the number of circulating neutrophils and macrophages. This helps protect against bacterial sepsis. In the current study, neutrophils played no role in microbiota-mediated enhancement of bacterial clearance from the lung and the amount of macrophages in the lung also was unchanged after microbiota depletion, probably a reflection of the low turnover rate of these cells in lung tissue (62). Enhanced clearance of bacteria from the lung did, however, depend on increased ROS production by alveolar macrophages via microbiota stimulation. Thus, in this study, functional reprogramming of innate cells was found to be important for enhanced innate immunity to bacterial infection rather than increased innate cell production. All of these studies fit with recent reevaluations of PRR function, positing that they play a more nuanced role in host physiology, acting as regulators of immune homeostasis and not purely as sensors of infection (6).

Much remains to be understood about the systemic influence of commensal bacteria on host defense against infection. The continued worldwide mortality caused by bacterial infection means that the widespread use of antibiotics will continue. However, in addition to increasing antibiotic resistance, antibiotic-mediated microbiota disruption could lead to increased susceptibility to bacterial infection because of the profound importance of commensal stimulation for innate responses to pathogens (4). Because of this, it is important to delineate whether all microbial groups within the microbiota are equal in their ability to program innate cell function to be able to develop therapeutic strategies that avoid those that are important for stimulating the antibacterial activity of innate cells. Furthermore, the wide-ranging influence of the commensal microbiota on the innate response to bacterial infection suggests that adaptive immunity to bacterial pathogens at extraintestinal sites will be similarly influenced. Deciphering the mechanistic basis for these effects could be of tremendous utility in the fight against infectious disease, as it could suggest novel strategies to enhance immune responses elicited by vaccines.

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